dicholormethane/trifluoroacetic acid (3:7) mixture. After 60 minutes the solution was concentrated. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). Yield 0.75g. Analysis. Calcd. for N, 0.28. Found: N, 0.10.

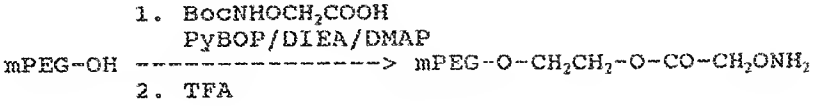
Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-NH-CO-CH_2-ONH_2$

$$mPEG\text{-}NH_2 \xrightarrow[2.\ TFA]{1.\ BocNHOCH_2COOH,\ PyBOP/DIEA} mPEG\text{-}O\text{-}CH_2CH_2\text{-}NH\text{-}CO\text{-}CH_2ONH_2$$

mPEG-5000-amine (2.0g, 0.4 mmol) and Boc-aminoxyacetic acid (0.2g, 1.05 mmol) were dissolved in 20ml dichloromethane. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (1.1g, 2mmol) was added followed by diisopropylethylamine (0.7ml, 3.9 mmol). The reaction was stirred for about 72 hours at room temperature after which time cold ether was added, and the resulting precipitate was collected by filtration, washed, and dried. Half of the collected precipitate was paced in a dicholormethane/trifluoroacetic acid (3:7) mixture. After 60 minutes the solution was concentrated. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). The resulting yellow precipitate was taken up in water and was treated with decolorizing carbon. After about 24 hr. the decolorizing carbon was filtered, and the clear filtrate was concentrated. The residue was dissolved in dichloromethane, dried with sodium sulfate, filtered, and the filtrate was

treated with cold ether. A white product was obtained. Yield 0.5g. IR: (C=0): 1676. Analysis. Calcd. for N, 0.55. Found: N, 0.50.

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-O-CO-CH_2-ONH_2$

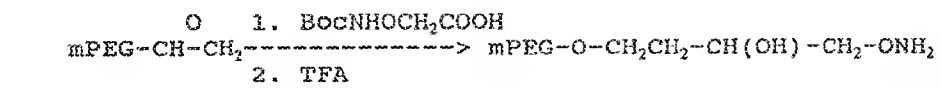



mPEG-5000-alcohol (2.0g, 0.4 mmol) and Boc-aminooxyacetic acid (0.2g, 1.05 mmol) were dissolved in 20 ml dichloromethane. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexaflurophosphate (1.1g, 2mmol) was added followed by diisopropylethylamine (0.7ml, 3.09 mmol). The reaction was stirred for about 2 hours at room temperature after which time dimethylaminopyridine (0.244g, 2 mmol) was added. After about four days cold ether was added, and the resulting precipitate was collected by filtration, washed, and dried. The precipitate was taken up in water and was treated with decolorizing carbon. After about 24 hr. the decolorizing carbon was filtered, and the clear filtrate was concentrated. The residue was dissolved in dichloromethane, dried with sodium sulfate, filtered, and the filtrate was treated with cold ether. Analysis. Calcd. for N, 0.28. Found: N, 0.14.

Half of the collected precipitate was placed in a dicholormethane/trifluoroacetic acid (2:1) mixture. After 30 minutes the solution was concentrated. The compound was further purified by gel filtration on a

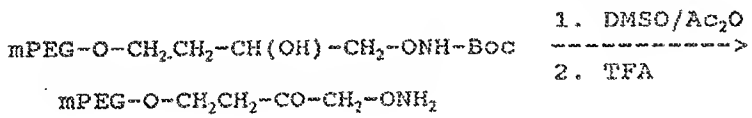
LH-20 column eluting with methanol/methylene chloride (5:1). Yield 0.3g. IR: (C=O): 1734.

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-CH(OH)-CH_2-ONH_2$



mPEG-5000-epoxide (1.0g, 0.2 mmol) was dissolved in 10ml 0.1M NaOH. t-Butyl N-hydroxycarbamate (0.53g, 4 mmol) was added. After running the reaction overnight the reaction mixture was extracted with dichloromethane. Sodium sulfate was added and was filtered. Cold ether was added the dichloromethane solution, and the resulting precipitate was collected by filtration, washed, and dried. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). The protected mPEG-derivative (0.25g) was placed in a dicholormethane/trifluoroacetic acid (1:1) mixture. After 30 minutes the solution was concentrated and taken up in dichloromethane. The compound was isolated by precipitation from ether. Yield 0.2g. IR: (O-H): 3447. Analysis. Calcd. for N, 0.28. Found: N, 0.21.

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-CO-CH_2-ONH_2$



mPEG-O-$CH_2CH_2$-CH(OH)-$CH_2$-ONH-Boc (0.3g, 0.6 mmol) was placed in 3 ml dry dimethyl sulfoxide followed by the addition of 2 ml dry acetic anhydride. The reaction went for about 24 hr. at room temperature after which time cold ether was added. The resulting precipitate was collected by filtration, washed and dried. The product was placed in a dicholormethane/trifluoroacetic acid (1:1) mixture. After 30 minutes the solution was concentrated. The compound was isolated by precipitation from cold ether. Yield 0.18g. IR: (C=0): 1698. Analysis. Calcd. for N, 0.28. Found: N, 0.20.

Synthesis of mPEG-Ornithine Semicarbazide

mPEG-5000-amine (5.0 g, 1. mmol) was dissolved in 5 ml dry methylene chloride and 10 ml dry dimethylformamide was added. Fmoc-Orn(Boc)-OPfp (3.1 g, 5 mmole) was added. After 1 hr. at room temperature the solution was concentrated. Water was added to the residue, and the solution was filtered, centrifugred, and filtered to remove the dispersed solid in the aqueous solution. The filtered aqueous solution was concentrated, and the residue was taken up in methylene chloride and was dried with sodium sulfate. The solution was filtered. The filtrate was treated with cold ether. The resulting precipitate was colleced by filtration, washed, and dried. Yield 3.3 g. IR: (C=0):1713, 1680. The Fmoc group was removed by treating the compound with 25% piperidine (in methylene chloride) for 30 min. Cold ether was added to the solution to precipitate the mPEG-derivative. The precipitate was collected, washed, and dried. The free alpha amino group was acetylated by dissolving 1.4 g of the mPEG-derivative

with 3 ml methylene chloride and adding 1 ml acetic anhydride. After about 1.7 hr. at room temperature the solution was concentrated. The resulting solid was treated with trifluoroacetic acid in methylene chloride (3:5) for 1 hr. at room temperature. The solvent was removed and resulting oil was taken up in methylene chloride and cold ether was added. The precipitate formed was collected, washed, and dried. Yield 1.1 g. IR: (C=O): 1685. The mPEG-derivative was dissolved in 3 ml dry methylene chloride and triethylamine (0.54 ml, 3.84 mmole) was added followed by 1 ml phosgene in toluene (1.92 mmole) and an additional 2 ml dry methylene chloride. The reaction went overnight at room temperature after which time the solvent was removed. The residue was dissolved in 3 ml dry methylene ch loride and 0.2 ml hydrazone (5.76 mmole) was added. Dry methanol was added until the solution became clear (3.4 ml). After 4 hr. at room temperature the solution was clarified by centrifugation and was concentrated. The compound was purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). Yield 0.7 g. IR: (C=O): 1675.

The invention having been described, the following examples are offered by way of illustration, not by way of limitation, of the subject invention.

## EXAMPLES

### Modification of EPO (Hydrazide Method)

In a typical experiment, EPO (0.5-1.0 mg) (obtained from Ortho Biotech) was placed in 100 mM sodium acetate, pH 5.6, total volume 0.786 ml.

Enough 10 mg/ml solution of sodium periodate was added to give a final concentration of sodium periodate at 10 mMol. The oxidation went for 30 min at 0°C in the dark after which time 0.33 ml 80 mMol $Na_2SO_3$ was added. After 5 min the solution was concentrated and washed three times with 100 mMol sodium acetate, pH 4.2 in a microconcentrator. After the final concentration the oxidized EPO solution was brought up to 1.0 ml with 100 mM sodium acetate. mPEG5000-hydrazide (50 mg) was added to the oxidized EPO. The mixture was stirred over night at room temperature. The mPEG5000-EPO was purified by gel filtration using a Sephacryl S-200-HR column (1 mm x 45 mm) eluting with a phosphate buffer containing 0.05% sodium azide. The amount of mPEG modifying EPO was determined by HPLC gel filtration using a either a Zorbax® GF-250 or GF-450 column using a 0.1 M phosphate buffer, pH 7. From 6-12 molecules mPEGs were found to be attached to each molecule of EPO.

Modification of EPO (Semicarbazide Method)

The same procedure as above for the hydrazide method was performed, except the reaction time for oxidation was decreased to 5 minutes and a decreased amount of mPEG-semicarbazide (10 mg) was used compared to the amount of mPEG-hydrazide (50 mg). Even with the decreased oxidation time and less mPEG added, more (about 18) mPEG molecules were attached to EPO. If longer oxidation times (15 min) and more mPEG-semicarbazide is added, around 30 mPEG molecules can be attached to EPO depending on the molecular weight of mPEG used. Thus mPEG-semicarbazide appears to be more reactive than mPEG-hydrazide and attaches

many more mPEG molecules to EPO than mPEG-hydrazide is able to under similar reaction conditions.

A comparison of the effect of modifying EPO with mPEG on either the carbohydrate groups or on the amino acid side chains is shown in Fig. 1. Analytical HPLC gel filtration conditions are the same as described above. The chromatogram of unmodified EPO is presented in Fig. 1a. A single peak with a retention time of 10.5 min is found. When EPO is modified with mPEG5000 on its carbohydrate groups (Fig. 1b), a single large peak with a retention time of 9.4 minutes is seen for the unpurified reaction product. When EPO is reacted with a succinimide ester of mPEG5000 which reacts with the side chain of lysine, a heterogeneous mixture of reaction products is obtained (peaks from 7.5 - 10.2 minutes). A similar heterogeneous pattern for mPEG modification using succinimide coupling to CSF-1, interleukin-2, and $\beta$-interferon has been found (U.S. Pat No. 4,847,325 and 4,9117,888). There are also more low molecular weight impurities present with succinimide coupling (Fig. 1c). Active ester coupling using succinimide derivatives of mPEG has been the preferred method for attaching mPEG to proteins [Nucci, M.L., Shorr, R., and Abuchowski, A. (1991) Adv. Drug Delivery Rev., 6, 133-151]. EPO was also derivatized with mPEG8500 using the above-described semicarbazide method, see Figure 4 for results of biological experiments. The above-described semicarbazide method was also used to obtain EPO modified with mPEG12000 (see Figure 3) and EPO modified with mPEG2000 (see Figure 10).

EPO also was modified with thiosemicarbazide, hydrazide carboxylate, and carbonic acid dihydrazide

derivatives of mPEG. These derivatives of mPEG performed like the semicarbazide derivatives of mPEG in that high levels of coupling mPEG to EPO could be obtained using these derivatives when compared to the hydrazide derivatives of mPEG. Other conditions for the oxidation of EPO can be used such as increased temperature, increased concentration of sodium periodate, and increased or decreased reaction times as long as these oxidation conditions do not impair the biological activity of EPO.

Large-Scale Modification of EPO (Semicarbazide or Carboxylate Hydrazide Method)

EPO (12.0mg) (obtained from Ortho Biotech) was placed in 100 mM sodium acetate, pH 5.5, total volume 1.8 ml. Sodium periodate (0.215 ml) at a concentration of 40 mg/ml was added. The oxidation went for 20 minutes at 0°C in the dark after which time 0.02 ml of ethylene glycol was added, and the admixture stirred for 10 minutes at 0°C. The oxidized-EPO was purified by gel filtration using a Sephadex® G-25 column (2.5 cm X 9 cm) and eluted with 100mM sodium acetate buffer, pH 4.3. Eluted oxidized EPO (10-11ml) was pooled. mPEG5000 semicarbazide (100 mg) was added to the purified oxidized EPO. The mixture was stirred overnight at room temperature. The mPEG-5000 EPO was purified by gel filtration using a Sephacryl® S-200-HR column eluting eith buffer consisting of 0.2M NaCl, 0.02 M sodium citrate, 0.025% sodium azide, pH 7.0.

The above modification was repeated using 200 mg of mPEG5000 semicarbazide. Reactivity was about 22 mPEG molecules per molecule of EPO.

The above modification was repeated employing half the amount of the reactants as specified

hereinabove, using 200 mg of carboxylate hydrazide. Reactivity was about 30 mPEG molecules per molecule of EPO.

Modification of EPO (Oxime Method)

A. mPEG-$CH_2CH_2$-NH-CO-$CH_2$-$ONH_2$

The same procedure as above for the large-scale semicarbazide method was performed. However, instead of addition of mPEG5000 semicarbazide, mPEG5000-CH2-CH2-NH-CO-$CH_2$-$ONH_2$ (50 mg) was admixed with 2.15 ml of oxidized EPO at room temperature overnight. The mPEG5000-EPO was purified by gel filtration using a Sephacryl® S-200-HR column eluting with buffer consisting of 0.2M NaCl, 0.02 M sodium citrate, 0.025% sodium azide, pH 7. About 31 molecules of mPEG5000 were found to be attached to each molecule of EPO as determined by HPLC gel filtration using a Phenomenex Biosep-Sec-S4000 column (30 cm X .017 cm). A minor fraction consisting of 25 molecules of mPEG-EPO was also isolated, and was used for biological testing. See Figures 16, 18 and 19.

B. mPEG-O-$CH_2CH_2$-NH-CO-$ONH_2$

The above modification of EPO was repeated using mPEG-O-$CH_2CH_2$-NH-CO-$ONH_2$. About 18-19 molecules of mPEG5000 were found to be attached to each molecule of PEG as determined using the methods as in modification A above. Biological data is shown in Figures 16, 18 and 19.

C. mPEG-O-$CH_2CH_2$-$ONH_2$

The above modification of EPO was repeated using mPEG-O-$CH_2CH_2$-$ONH_2$. About 17 molecules of mPEG5000 were found to be attached to each molecule of PEG as

determined using the methods as in modification A above. Two minor fractions of 22mPEG, 12mPEG were also isolated(Figure 17, 18, 19).

D. $mPEG-O-CH_2CH_2-CO-ONH_2$

The above modification of EPO is repeated using the PEG-oxime derivative $mPEG-O-CH_2CH_2-CO-ONH_2$. About 3 molecules of mPEG5000 were found to be attached to each molecule of EPO, as determined using the methods as for modification A above.

E. $mPEG-O-CH_2CH_2-CH(OH)-CH_2-ONH_2$

The above modification of EPO is repeated using the PEG-oxime derivative $mPEG-O-CH_2CH_2-CH(OH)-CH_2-ONH_2$. About 31 molecules of mPEG5000 were found to be attached to each molecule of EPO, as determined using the methods as for modification A above.

F. $mPEG-O-CH_2CH_2-NH-CS-ONH_2$

The above modification of EPO is repeated using the PEG-oxime derivative $mPEG-O-CH_2CH_2-NH-CS-ONH_2$. About 4 molecules of mPEG5000 were found to be attached to each molecule of EPO, as determined using the methods as for modification A above.

Biological Activity of mPEG-EPO

The mPEG-EPO derivatives were assayed for biological activity in vivo by measuring the increase in erythrocytes generated after injection of the modified protein (Egrie, J.C., Strickland, T.W., Lane, J., Aoki, K., Cohen, A.M., Smalling, R., Trail, G., Lin, F.K., Browne, J.K., and Hines, D.K. (1986) Immunobiol. 172: 213-224). Briefly, mice (female CD-1, eight weeks old) were injected either

intraperitoneally or subcutaneously with 0.4 µg protein once a day for two consecutive days. Blood was withdrawn on predetermined days for hematocrit readings.

The in vivo biological activities (hematocrit levels) of the mPEG-EPOs linked via hydrazide derivatives of mPEG is presented in Figures 2, 4 and 5 and Tables I and II. To summarize the findings, Figures 2 and 5 show that the optimal number of mPEG coupling is not obvious and has to be determined by synthesis and biological testing in order to give the best mPEG-EPO. Figure 4 compares mPEG coupling using hydrazide and semicarbazide linkers. Higher and longer hematocrit levels for mPEG-EPO could be obtained using the semicarbazide linker. The observed results are due, in part, to the higher level of incorporation of mPEG which could be obtained using the semicarbazide linker. Table I shows the biological activity of the mPEG-EPOs as a function of the number of mPEGs incorporated and the molecular weight of mPEG used.

Table I summarizes the biological activity of different hydrazide mPEG-EPOs comparing molecular weight of mPEG used and the amount of mPEG coupled.

Table II shows a comparison of the different mPEG hydrazide linkers used. Table II summarizes the biological activities of EPO modified with different mPEG5000-hydrazide derivatives where optimal amounts of mPEG were incorporated. Not all the carbohydrate mPEG-derivatives give the same biological activity when coupled to EPO due to the inability to sufficiently couple an optimal amount of mPEG or other factors. Biological activities are the best values obtained for each linker.

TABLE I

Table I: Biological Activity of Different mPEG-EPOs

| #mPEGs Coupled * | Molecular Weight mPEG | Max. Hematocrit (%)** | Duration of Activity Relative to EPO (Days)*** |
|---|---|---|---|
| 15 | 2000 | 60 | 14 |
| 8 # | 5000 | 53 | 8 |
| 12 # | 5000 | 51 | 7 |
| 18 | 5000 | 61 | 19 |
| 22 | 5000 | 61 | 20 |
| 24 | 5000 | 59 | 13 |
| 28 | 5000 | 54 | 14 |
| 17 | 8000 | 59 | 16 |
| 12 # | 8500 | 54 | 7 |
| 20 | 8500 | 58 | 14 |
| 34 | 8500 | 47 | 7 |
| 8 # | 12000 | 54 | 7 |
| 14 | 12000 | 53 | 11 |
| 29 | 12000 | 51 | 7 |

* As Determined by Size Exclusion Chromatography

** Biological Assay Described in Experimental Section

*** Days from EPO Maximum Hematocrit Level (46 on Day 4) Required to Reach EPO's Maximum Hematocrit Level After Attaining Its Own Maximum Hematocrit Level

\# Hydrazide Linker Used. All Others Used Semicarbazide Linker.

Table II: Biological Activity of mPEG5000-EPOs Comparing Different Carbohydrate Modifying mPEG-Linkers

| mPEG-Linker | | Max. Hematocrit (%) * | Duration of Activity Relative to EPO (Days)** |
|---|---|---|---|
| $CH_3$-$(OCH_2CH_2)_n$-O-$CH_2$-CO-$NHNH_2$ | (Hydrazide) | 54 | 8 |
| $CH_3$-$(OCH_2CH_2)_n$-NH-CO-$NHNH_2$ | (Semicarbazide) | 61 | 20 |
| $CH_3$-$(OCH_2CH_2)_n$-NH-CS-$NHNH_2$ | (Thiosemicarbazide) | 59 | 14 |
| $CH_3$-$(OCH_2CH_2)_n$-NH-CO-NHNH-CO-$NHNH_2$ | (Carbonic Acid Dihydrazide) | 57 | 12 |
| $CH_3$-$(OCH_2CH_2)_n$-O-CO-$NHNH_2$ | (Hydrazide Carboxylate, 22 mPEGs) | 60 | |
| $CH_3$-$(OCH_2CH_2)_n$-NH-CO-$C_6H_4$-$NHNH_2$ | (Arylhydrazide) | 52 | 7 |

* Biological Assay Described in Experimental Section
** Days from EPO Maximum Hematocrit Level (46% on Day 4) Required to Reach EPO's Maximum Hematocrit Level After Attaining Its Own Maximum Hematocrit Level

The hematocrit levels for EPO and mPEG5000-EPOs in mice are presented in Fig. 2. The 12PEG-EPO was made by coupling mPEG5000-hydrazide and reflects the maximum incorporation which could be achieved by this mPEG derivative under the experimental conditions given above. The 18PEG and 28PEG EPOs were made by coupling with mPEG5000-semicarbazide. The semicarbazide derivatives of mPEG result in much better biological activity than the hydrazide derivatives of mPEG due to the larger amounts of mPEG which can be incorporated using this mPEG derivative. All three mPEG5000-EPOs show increased maximum and prolonged activity when compared to native EPO. Thus modification of a protein's carbohydrate groups with PEG can yield a much more potent therapeutic protein.

For additional data on the effects of various mPEG hydrazide-modified EPOs employed in the experiments on hematocrit levels, see Figures 8-15. The mPEG modified EPO employed in the experiments depicted in Figures 8-15 were prepared using the appropriate water-soluble polymer reagent essentially as described for the other mPEG modified EPO molecules used the experiments depicted in Figures 2-5.

The in vivo biological activities (hematocrit levels) of the mPEG-EPOs linked via oxime-forming derivatives of mPEG is presented in Figures 16 and 17. To summarize, Figure 16 compares mPEG coupling using mPEG-O-CH2CH2-NH-CO-ONH2 ("A") and mPEG-O-CH2CH2-NH-CO-CH2-ONH2 ("C") linkers. Higher hematocrit levels of mPEG-EPO could be obtained using the "A" linker (corresponding to Formula XXX herein) having 18 mPEG molecules per molecule of EPO as compared to the "C" linker (corresponding to Formula

XXXIII herein) having 31 mPEG molecules per molecule of EPO. Also notable was the higher hematocrit activity of the "C" linker (Formula XXXIII herein) having 25 mPEG molecules per molecule of EPO as compared to the same linker having 31 molecules of mPEG per molecule of EPO.

Figure 17 compares mPEG coupling using mPEG-O-CH2CH2-ONH2 (formula XXIII) ("B") linker at 22, 17, and 12 mPEG molecules per molecule of EPO. Highest hematocrit levels are obtained at the lowest degree of pegylation, and hematocrit was decreased inversely proportional to degree of pegylation. In all mPEG linkers using mPEG-O-CH2CH2-ONH2 oxime derivative however, hematocrits were higher and of increased duration as compared to native EPO.

Especially noteworthy is the biological activity of the 12mPEG-EPO of formula XXIII. Hydrazide derivitized 12 mPEG-EPO produces neither the degree nor duration of hematocrit elevation as that of the oxylamine derivitized EPO.

Antibody binding to mPEG-EPO

The antigenicity of the mPEG-EPOs was determined by using the Clinigen™ erythropoietin (EPO) EIA test kit. Briefly, the assay consists of a micro titre plate coated with a monoclonal antibody to EPO. EPO or mPEG-EPO is allowed to interact with the coated plate. After washing the plate a labeled polyclonal antibody to EPO is incubated on the plate. After substrate development the plate is read.

The results of the ELISA assay for hydrazide derivatized EPO, are presented in Figure 3. The mPEG-EPOs are presented as the approximate number of mPEGs attached for a given molecular weight of mPEG.

For example, 12PEG-5k means about 12 mPEG molecules of a molecular weight of about 5000 were coupled to each molecule of EPO. The data indicates that as the number of mPEGs coupled to EPO are increased, the antigenicity of the protein is decreased. Similarly as the molecular weight of mPEG is increased, the antigenicity, i.e. the binding of the antibody, of the modified EPO also is decreased. Reacting oxidized EPO with a hydrazide derivative of mPEG did not reach the high coupling levels seen with semicarbazide, thiosemicarbazide, and carbonic acid dihydrazide PEG derivatives and thus could not give the large decreases in immunogenicity as seen with these other mPEG derivatives. Decreasing the antigenicity of a protein correlates to a decrease in the immunogenicity of a protein as well. Thus mPEG-EPO coupled to the carbohydrate groups of EPO may reduce any potential immunogenicity related to the protein with those derivatives of mPEG able to be coupled at high levels being the most effective.

For additional ELISA data with mPEG hydrazide derivatives see Figure 6.

The results of the ELISA assay for oxime derivatized EPO are presented in Figure 18. The data indicates that as the number of mPEGs coupled to EPO are increased, the antigenicity of the protein is decreased. Reacting oxidized EPO with a linker that resulted in comparatively low coupling levels (18 PEG-A, 17 PEG-B, 12 PEG-B) did not give the large decreases in immunogencity as seen with the comparatively high coupling level formulations (22 PEG-B, 25 PEG-C, and 31 PEG-C. Note that these differences in a linker's ability to decrease immunogenicity appear to be determined largely based

on the coupling level (e.g. compare 12 PEG-B and 22PEG-B). Thus, mPEG coupled to carbohydrate groups of EPO through oxime linkages may reduce potential immunogencity related to the protein, with those derivatives of mPEG able to be coupled at high levels being the most effective.

Modification of Horseradish Peroxidase: Comparison of Hydrazide Versus Semicarbazide Coupling

Horseradish peroxidase (HRP) is a glycoprotein enzyme (oxido-reductase). HRP was modified with either mPEG5000-hydrazide or mPEG5000-semicarbazide in order to see whether another glycoprotein besides EPO could show the difference in modification between the two different carbohydrate modification reagents. In a typical experiment, horseradish peroxidase (2 mg) was placed in 100 mM sodium acetate, pH 5.6, total volume 0.8 ml. Enough 10 mg/ml solution of sodium periodate was added to give a final concentration of sodium periodate at 10 mMol. The oxidation went for 15 min at 0°C in the dark, after which time 0.33 ml 80 mMol $Na_2SO_3$ was added. After 5 minutes, the solution was concentrated and washed three times with 100 mMol sodium acetate, pH 4.2, in a micro concentrator. The oxidized horseradish peroxidase solution was then split in half, with one half receiving mg PEG5000-hydrazide and the other half receiving 30 mg mPEG5000-semicarbazide. The two oxidized horseradish peroxidase solutions were then stirred overnight at room temperature. The extent of PEG modification was determined by HPLC gel filtration using a Zorbax™ GF-250 column using a 0.1 M phosphate buffer, pH 7. The mPEG5000-hydrazide modified horseradish peroxidase had approximately 7

PEG molecules/HRP; the mPEG5000-semicarbazide modified horseradish peroxidase had approximately 19 PEG molecules/HRP. Thus modification of horseradish peroxidase with PEG being attached to its carbohydrate groups is more effective using a semicarbazide derivative of PEG than a hydrazide under the same experimental conditions.

Half-Life Determinations

Half-Life experiments were done in male Sprague-Dawley rats weighing about 0.3 kg. Three rats were used for each compound. The experimental details are as follows. Each rat was injected IV (intravenously) with 1 μg EPO or mPEG-EPO. For the hydrazide derivatized mPEG-EPO, the mPEG-EPO used was mPEG500 semicarbazide-18, prepared essentially as described in the section above on EPO modification with semicarbazides. Blood was withdrawn from each rat at 2, 5, 15, 45, 90 minutes and 3, 6, 24, 48, 54 hour time points. The blood was collected in heparinized tubes, and the plasma was isolated. The isolated plasma was tested for EPO biological activity in an EPO dependent cell proliferation assay. The in vitro assay employed used an FDC-P1/ER cell line. This murine cell line incorporates the EPO receptor and is dependent on EPO for growth. The assay was performed as follows. The cells were grown (106/ml) in the absence of EPO for 24 hr after which time either EPO or mPEG-EPO at different concentrations is added to the cells. The cells were incubated for 42 hr, and then tritiated thymidine was added to the cells. After 6 hr the cells were harvested and counted. Cell growth was determined by the increased up-take of thymidine. Results are given in figure 7.

The EPO dependent cell proliferation assay was performed as described above using oxime-derivatized mPEG-EPO. Results are given in Figure 19.

In Vivo Assay: Anemic Mouse Model

In this assay mice are rendered anemic by injections for five consecutive days with TNF-alpha. To overcome the anemia the mice were injected SC (subcutaneously) with either EPO or mPEG-EPO (at 0.03 μg/dose) over the same five days or on just two of the five days that the mice receive TNF-alpha. Results are given in figure 15.

Equivalents

All publications and patents mentioned in the above specification are herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A compound having the formula:

wherein X is O or S; Q is selected from the group consisting of $-NHNH_2$, and $-C_6H_4-NHNH_2$; and Y is selected from the group consisting of -O-, $-OCH_2-$, -NH-, -NHNH-, $-O-CO-CH_2CH_2-$ and -NHCO-N-NHNH-; and P is a water-soluble polymer.

2. A compound according to claim 1, said compound belonging to the group consisting of,

a compound having the formula:

(I) $P-O-CH_2-CO-NHNH_2$,

a compound having the formula:

(II) $P-O-CO-NHNH_2$,

a compound having the formula:

(III) $P-NH-CO-NHNH_2$

a compound having the formula:

(IV) $P-NH-CS-NHNH_2$

a compound having the formula:

(V) $P-NHCO-N-NHNHCO-NHNH_2$

a compound having the formula:

(VI) $P-NHNHCONHNH_2$,

a compound having the formula:
(VII) P-NHNHCSNHNH$_2$,

a compound having the formula:
(VIII) P-NH-CO-$C_6H_4$-NHNH$_2$, and

a compound having the formula:
(IX) P-O-CO-$CH_2CH_2$-CO-NHNH$_2$.

3. A compound according to claim 2, said compound having the formula:

(II) P-O-CO-NHNH$_2$.

4. A compound according to claim 2, said compound having the formula:
(III) P-NH-CO-NHNH$_2$.

5. A compound according to claim 2, said compound having the formula:

(IV) P-NH-CS-NHNH$_2$.

6. A compound according to claim 2, said compound having the formula:
(V) P-NHCO-N-NHNHCO-NHNH$_2$.

7. A compound according to Claim 2, wherein said polymer is selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, polyoxyethylated polyols,